Attorney Docket No. P58126US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of Metin COLPAN

Application No.: 08/796,040 Art Unit: 1623

Filed: February 5, 1997 Examiner: Lawrence E. Crane

For: DEVICE AND A PROCESS FOR THE ISOLATION OF NUCLEIC ACID

SUBSTITUTE APPEAL BRIEF

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

The present substitute brief on appeal is submitted pursuant to the Order mailed June 29, 2006, and in response to the Notification of Non-Compliant Appeal Brief, mailed August 24, 2006.

Appellant wishes to thank Primaruy Examiner Lawrence E. Crane for the courteous consideration rendered appellant's undersigned representative during telephone discussions on or about July 10 and 12, and August 14 and 16, 2006. The discussions concerned compliance with the Order mailed June 29, 2006, by the PTO Board of Patent Appeals and Interferences.

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(1) Real Party in Interest

The real parties in interest is Qiagen GmbH, a corporation of Germany, pursuant to assignments recorded in the United States Patent and Trademark Office (PTO) on August 2, 1994, at reel/frame 7121/0455.

(2) Related Appeals and Interferences.

There are no other appeals or interferences.

(3) Status of Claims.

Claims 1-119 were canceled. Claims 120-138 (Claims Appendix, *infra*) are pending and finally rejected under 35 USC 103(a) as being allegedly unpatentable over US 5,057,426 (Henco), in view of US 5,075,430 (Little) and, further, in view of *International Dictionary of Medicine and Biology, 1*, 1986, page 522 (*International Dictionary*), and *Nucleic Acid Hybridisation - A Practical Approach*, 1985, pages 64, 65, and 235 (Hames).

(4) Status of Amendments.

There are no amendments after final rejection. An information disclosure statement was filed after final rejection, i.e., on March 10, 2005. Appellant has received no communication from the PTO concerning the information disclosure statement.

(5) Summary of Claimed Subject Matter.

The presently claimed invention provides a process for isolating and purifying nucleic acids found in cells, i.e., using the cells as the starting materials (application page 1, lines 1-3). The process involves two chromatographic purification (adsorbing-desorbing) stages, operated in tandem; whereby, purified nucleic-acid-containing material obtained in the first stage is applied to the second stage, directly, as it comes from the first stage.

In the first stage cells containing the nucleic acids to be isolated are digested, cell debris is removed, and the nucleic acids are adsorbed on anion-exchange material (application page 5, lines 7-36) and, subsequently, desorbed from the anion-exchange material; adsorption of the nucleic acids being effected in the presence of a buffer solution (Abstract, lines 7-8) under conditions of lower salt concentration than the buffer solution enabling the nucleic acids to be desorbed from the anion-exchange material (application page 6, lines 7-11).

In the second stage the nucleic-acids desorbed from the anion-exchange material, still in the higher-salt desorption buffer, are applied and adsorbed onto a mineral support (application page 8, lines 1-4), followed by desorbing the nucleic acids from the mineral support using a buffer solution having a lower ionic strength than the buffer in which the nucleic acids were applied (to the mineral support) (Abstract, lines 17-19).

The combination of adjusting the ionic strength of adsorption and desorption conditions with the adsorbing materials used in the two stages (i.e., anion-exchange material and mineral support) in accordance with the presently claimed process, allows the *desorbing* buffer in the first stage to act as the *adsorbing* buffer in the second stage. Preferably, the anion-exchange material is based on a

support matrix (porous or non-porous) of surface-modified agarose, dextran, cellulose, acrylic amide, poly(vinyl alcohol), polystyrene, glass, aluminum oxide, titanium dioxide, zirconium dioxide, or, silica gel (application page 5, lines 17-21 and 24-28; page 12, lines 19-24; original claim 13). The anion-exchange material is preferably comprised of a porous or non-porous material (application page 5, lines 24-28; original claim 14) having a particle size of 1-250 μm (application page 5, lines 32-33; original claim 14); more preferably the particle size is 10-30 μm (original claim 14).

Preferred support materials are silica gel, glass, zeolite, aluminum oxide, titanium dioxide, zirconium dioxide, kaolin, diatomacae, or silica glass (application page 12, lines 29-32).

Preferably, each of the stages includes a "conditioning" step (i.e., between the adsorbing and desorbing steps), in order to optimize yields (application page 6, lines 23-26). A particularly preferred conditioning solution used in the second stage (i.e., applied to the mineral support between adsorbing and desorbing steps) corresponds to an ionic strength of about 1.5 M sodium perchlorate at a pH of approximately 5 (application page 6, lines 30-33).

The presently claimed process provides unique advantages for the purification of nucleic acids in the recited tandem *order* of using the "anion-exchange material" and "mineral support," that is, the exchange material, first, followed by the mineral support. Certain impurities, while being adsorbed to, and eluted from, the anion-exchange material along with the nucleic acids are incapable of adsorbing to the downstream mineral support under the same ionic conditions under which eluting from the anion-exchange material occurred. In other words, the buffer of higher ionic strength used to elute nucleic acids from the anion-exchange material not only allows for adsorption of the eluted

nucleic acids to the mineral support; it does not provide conditions for adsorbing the impurities to the mineral support (application page 9, lines 3-10).

(6) Grounds of Rejection to Be Reviewed on Appeal.

Whether claims 120-138 were incorrectly rejected under 35 USC 103(a) based on the combined teachings of Henco, Little, *International Dictionary*, and Hames.

(7) Argument.

The final rejection of claims 120-138 under 35 USC 103(a) (based on the combined teachings of Henco, Little, *International Dictionary*, and Hames) is both factually incorrect and legally erroneous. As explained in detail, below: the rejection of claims 120-138 cannot be sustained because (A) at least one feature (limitation) on all the rejected claims is neither taught nor suggested in the cited references, (B) the rejection is based on prohibited hindsight reconstruction, (C) the rejection fails to apply/satisfy the correct legal standards for analysis under §103(a), and (D) the rejection reads non-existing teachings into the cited references; further, the rejection of each of claims 122-126 and 129-136, independently, cannot be sustained because each relies on "phantom" prior art.

(A) Claims 120 - 138

The rejection is fatally defective because the cited references, taking the teachings of each reference as a whole, neither describe nor suggest a process that contains process step "c)," as recited in the present claims. That is, starting with the nucleic-acid containing buffer-solution product of anion exchange separation/purification of stage 1, the process step of

> c) adsorbing the separation/purified nucleic acids in the second buffer solution onto the surface of a mineral support material

(claim 120). In other words, the cited prior art does teach or suggest obtaining a solution of "twice purified nucleic acids" in a 2-stage process of (stage i) anion exchange extraction followed by (stage ii) further purifying the once-purified nucleic acids by inorganic solid phase extraction.

As broadly claimed (in claim 120) the instant invention is a process whereby "twice purified nucleic acids" are obtained from cells through the use of *two* separation/purification *stages* – stages "i)" and "ii)" recited in claim 20 – each stage involving two process steps.

In stage i): Cells are digested, the cell debris removed, and the nucleic-acid containing residue applied to anion exchanger material, under conditions that selectively bind the nucleic acids to the anion exchanger – freeing the nucleic acids from, *i.a.*, cell proteins. The protein-free nucleic acids are, then, washed out of the anion exchanger with a buffer solution "effecting [once] purified nucleic acids in the . . . buffer solution" (claim 20) – the product of stage i).

In stage ii): The one-purified nucleic acids in the buffer solution, from stage i), are applied to a mineral support, under conditions that specifically bind the nucleic acids to the mineral support. The nucleic acids are, then, washed from the mineral support "effecting twice purified nucleic acids" (claim 20).

Reliance on Henco and Little to meet the present claims is misplaced.

The process according to the instant claims saliently differs from Henco in that steps c) and d) of the present claims are neither taught nor suggested. Henco contains no motivation to modify the process disclosed therein by the steps c) and d) of the present claims. No hint is given in Henco that (i) an increase in salt concentration should be effected in the sample fraction, nor is there any hint that (ii) such a fraction should be subsequently treated by application to a mineral support material in order to bind thereto the nucleic acid contained in the fraction, nor is there any hint to (iii) subsequently elute the substrate-bound nucleic acids using a buffer having very low ionic strength.

Little provides no teaching or suggestion to supply the salient deficiencies in Henco. Almost the same distinction with Henco applies with regard to the distinguishing Little from the presently claimed process. Appellant could not find any teaching or suggestion in Little for using purified/separated nucleic acids as starting materials. Therefore, there is indeed no motivation to combine the two documents, either in modifying Little according to Henco or in modifying Henco according to Little; or that any motivation is provided in the art to look to Henco or Little as suggested by the statement of rejection.

Appellant respectfully submits that the combination of Henco and Little is overly simplistic. Again, Henco discloses purification of nucleic acids by an anion exchange treatment or an anion exchange separation process. The key features are binding the nucleic acid at low ionic strength and eluting the nucleic acids at concentrations in the range of 2 M salt in the buffer (the number can be derived from Fig. 4 of the specification of Henco). No use of any material for being a chaotropic salt is disclosed or suggested in Henco.

According to the statement of rejection, the "choice of a specific chaotropic agent to be included in an elution buffer is a variation in chromatographic procedure which statement of rejection asserts is clearly within the perview [*sic*] of the ordinary practitioner unless appellant has shown unexpected results" (Examiner's answer, page 13). The alleged "variation in chromatographic procedure" being within the knowledge "of the ordinary practitioner" merely points out that the skilled artisan would have known *how* to vary the chromatographic procedure taught in the prior art had the skilled artisan thought up the *idea* of doing so, in the first place. With all due respect, the statement of rejection's argument fails to take into account that *invention* comprises both

the *idea* of the invention and the *means* to achieve that idea. *In re Cocer*, 175 USPQ 26 (CCPA 1972). Both the idea and means to achieve the idea must be evidenced in the prior art in order to demonstrate lack of patentability. *Id.* That a difference with the prior art amounts to an alleged "optimal condition" is "not a substitute for some teaching or suggestion supporting an obviousness rejection." *In re Rijckaert*, 28 USPQ 2d 1955, 1957 (Fed. Cir. 1993). Again, both the *idea* and the *means* to achieve the idea must be evidenced in the prior art in order to show obviousness. *In re Hoffman*, 37 USPQ 222 (CCPA 1938). "That which is within the capabilities of one skilled in the art is not synonymous with obviousness [citations omitted]. *Ex parte Levengood*, 28 USPQ 2d 1300, 1302 (Bd. Pat. App. & Inter. 1993). Whether or not Little accidently uses a substance that happens to be, assuming arguendo, a chaotropic agent, but does not use it for that purpose, does not suggest use of the material as a chaotropic agent to one of ordinary skill in the art. *Minnesota Mining & Manufacturing Co., supra.*

On the other hand, Little binds nucleic acids from a solution having a very high content of salts, especially chaotropic salts.

The skilled artisan would not have had any incentive to even increase the "high" salt concentration obtained after Henco's process after reading Little's disclosure. That optional desalting is taught in Henco by the procedures disclosed therein is not disputed, but it fails to support the allegation that the skilled artisan would have been motivated to rely on Little's process in order to "desalt" Henco's sample. According to Henco, if desired, the skilled artisan would, regardless of the circumstance, try to reduce the salt content; either by applying a salt concentration, as low as

possible, in the eluting step or by trying to desalt the sample by well known conservative methods, such as dialysis or gel permeation chromatography.

By no means however, would the skilled artisan ever consider, as opposed to getting rid of the salt, actually *increasing* after elution the salt content of the sample in Henco's process in order to obtain a sample having a very, very high salt concentration, as required in Little. The fact that, in accordance with the presently claimed invention, there is performed the step of increasing the salt content after Henco's process, in order to be able to employ process steps as disclosed in Little, may be regarded as a key unexpected step of steps as disclosed in the present invention.

The statement of rejection maintains that Little contains motivation to substitute the three desalting methods used in Henco (column 7, lines 44 to 46) with the silica separation method according to Little. The statement of rejection is mistaken.

Henco **starts** with DNA having a relatively low concentration of salt, which is not comparable with the situation Little addresses in the introductory portion of his disclosure. The DNA fractions dealt with in the paragraph cited by the statement of rejection are obtained after a cesium chloride gradient centrifugation. With respect to the samples which would be obtained in "too high a dilution," appellant submits that Henco teaches a method for separating DNA, wherein the DNA is not highly diluted in the eluate obtained from the method. Since the DNA is first absorbed on the chromatographic matrix and is afterwards desorbed in one elution step, the concentration of DNA is considerably high in the feral eluate. By analogy, therefore, Little's separation would be considered by the skilled person as an alternative *separation* method for isolating DNA; not as a mere substitute desalting step.

On the other hand, the method of the presently claimed invention utilizes, for the first time, the *effect* of silica disclosed in Little for such desalting steps. Originally, Little was not at all dealing with a desalting method, but with a separation method starting with highly concentrated salt solutions. This is evident from column 2, line 17 et seq. of Little, were it is stated: "This invention is directed to a process for the *purification* of plasmid and other DNA, both single-stranded and double-stranded, by immobilizing the DNA onto diatomaceous earth particles and eluting the DNA with water or low salt buffer (emphasis added)."

Therefore, the skilled artisan would not have considered using the procedure of Little as the optional desalting step of Henco. The argument made in the statement of rejection is a matter of hindsight; picking out features of the claimed process and trying to find the features in some piece of prior art. "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention." *In re Fine*, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988). Little is not concerned in any way with desalting of a sample. Little is concerned with purification of DNA found in a high-salt solution. Henco, however, does not yield such a sample having nucleic acid in a high salt environment.

The statement of rejection takes out of context certain statements made in Little; which distorts what is, actually, described by the reference. That is, Little states "the invention is directed generally to the immobilization of DNA onto diatomaceous earth which comprises contacting the DNA with the diatomaceous earth in the presence of a chaotropic agent" (Little column 2, lines 32-35). Similar statements are made, elsewhere, in the reference. The statement of rejection

characterizes these references in a manner that makes it *appear* as if Little encompasses (that is, *contemplated*) using *isolated* DNA as a *starting material*.

On the contrary, Little was concerned with the *desire* "to rapidly and inexpensively *separate* and purify DNA that was also amenable to scale-up" (Little column 1, lines 66-67). Little contemplated purifying DNA from "bacterial lysates" (Little column 1, lines 11-12); "plasmid DNA *from mini-prep lysates* can be purified using the process of the present invention" (Little column 5, lines 43-44), "this example illustrates that DNA can be purified *from bacterial lysates* independently of the method used to prepare the DNA and *without prior phenol extraction to remove proteins*" (Little, example 1) "the isolation of supercoiled DNA *from an agarose gel* by binding onto diatomaceous earth" (Little example 4), "nucleoside from triphosphates *are effectively removed from radiolabeling reactions* by the process of the present invention" (Little example 5), "the removal of linkers *from cloning reactions* using the process of the present invention" (Little example 6). Accordingly, Little contemplated, and described, a process that would address the problem whereby the "purification of plasmid DNA from bacterial lysates is a rate-limiting and time-consuming step in molecular biology" (Little column 1, lines 11-13), and fulfilled the objective whereby "a method was still desired *to rapidly and inexpensively* separate and purify DNA that was also amenable to scale-up" (Little column 1, lines 66-68).

As a result, the desirability (that is, motivation) provided by Little was to develop a process for isolated and purifying DNA that was more *rapid* than known methods. This motivation would not have led one of ordinary skill in the art to combine Little with Henco since it would not have sped up the process of either Little or Henco, at all; in fact, it would have increased the time over and

above that needed to perform either the Henco process or the Little process. If there were any motivation, it would have been to *replace* the Henco method, entirely, with the Little method; which, also, would have effected the optional *desalting* step taught by Henco.

The rejection uses impermissible *hindsight*; that is, by selectively picking and choosing from Little's teachings in a manner that fails to appreciate Little, as a whole.

> It is impermissible within the framework of §103 to pick and choose from any one reference only so much of it as will support a given position, to the exclusion of other parts necessary to the full appreciate of what such reference fairly suggests to one of ordinary skill in the art.

In re Hedges, 228 USPQ 685, 687 (Fed. Cir. 1986).

Furthermore, the suggested combination would destroy the invention upon which Little was based; that is, for example, a *one*-step procedure to save time. A reference cannot be used (under § 103) to show obviousness in a manner that destroys the invention on which the reference is based. *In re Gordon*, 221 USPQ 1125 (Fed. Cir. 1984).

The rejection, also, is fatally defective because reliance on *International Dictionary* and Hames is misplaced.

International Dictionary and Hames are relied upon to allegedly show that Henco describes the use of "chaotropic" salts and, therefore, is allegedly appropriately combined with the teachings of Little (cited in the statement of rejection) which teaches the binding of nucleic acids found in a solution having a high concentration of "chaotropic" salts. . . .

International Dictionary and Hames add nothing to cure the fatal deficiencies found in the Henco and Little references . . . . The statement of rejection relies on the two newly cited references to allegedly show that materials disclosed in the previously cited prior art fall within the definition of "chaotropic agents." However, even assuming, arguendo, the statement of rejection is correct, the statement of rejection acknowledges that the "Henco reference does not make specific reference to a chaotropic agent." Whether or not, however, materials described by Henco accidentally fall within a broad definition of "chaotropic agent," Henco neither teaches nor suggests use of these materials for their allegedly *chaotropic* function. Therefore, even should the newly cited references show that materials described in the originally cited prior art fall within the definition of chaotropic agent, there remains no teaching or suggestion in the prior art for using these materials as chaotropic agents; and, the statement of rejection's argument that they might function as such provides no reason or motivation for one of ordinary skill in the art to combine the materials in the manner presently claimed. Claims do not read on the prior art if "chemicals, although present in the prior art, were used for other non- . . . [claimed] functions and did not [perform the claimed function] . . . as . . . understood from the . . . specification." *Minnesota Mining & Manufacturing Co. v. Johnson & Johnson Orthopaedics, Inc.*, 24 USPQ 2d 1321, 1327 (Fed. Cir. 1992).

According to the statement of rejection, Appellant's arguments are not persuasive for failing "to provide a clear statement of how the combination of Henco et al. and Little has in any way destroyed the invention of Little as delineated by the claims found at the end of Little." The statement of rejection's reliance on the "claims found at the end of Little" as to what Little taught to one of ordinary skill in the art is misplaced. It is well-established that a patent's *claims* are no

measure of what a patent discloses for prior art purposes of 35 U.S.C. 102 and 103. *In re Benno*, 226 USPQ 683 (Fed. Cir. 1985). Indeed, it is the teachings of Little, as a whole, not merely those of Little's claims that must be applied to the presently claimed invention for purposes of analysis under §103 of the statute.

The statement of rejection mischaracterizes Appellant's arguments as based on destroying "the motivation provided by Henco." Appellant made no argument about destroying "the motivation provided by Henco"; it is the motivation alleged by the statement of rejection to be found in the prior art, which Appellant disputes. It is the combined teachings of the prior art, taken as a whole, which must be considered in an obviousness analysis. *Ryko Manufacturing Co. v. Nu-Star, Inc.*, 21 USPQ 2d 1053 (Fed. Cir. 1991). Therefore, increasing the salt concentration in accordance with the teachings of Little is *not* "irrelevant to the question of motivation" to combine the prior art, contrary to the statement of rejection's argument.

According to present claim 125 a further washing step can be introduced between step c) and d) by applying an aqueous alcoholic solution. According to claim 136, which is dependent from claim 125, the alcoholic solution may include 1 to 7 M sodium perchlorate, 1 to 7 M guanidine hydrochloride, 1 to 6 sodium iodide or 1 M sodium iodide or 1 M sodium chloride in 20 % ethanol, propanol, iso-propanol, butanol, poly(ethylene glycol) or mixtures thereof.

It is no doubt true that the salts sodium perchlorate, guanidine hydrochloride, and sodium iodide belong to the group of chaotropic substances or chaotropes according to the so-called Hofmeister series. The counterpart of these chaotropes are the so-called kosmotropes.

The terms 'chaotrope' (disorder-maker) and 'kosmotrope' (order-maker) originally denoted solutes that stabilized, or destabilized respectively, proteins and membranes. Later they referred to the apparently correlating property of increasing, or decreasing respectively, the structuring of water.

Large singly charged ions, with low charge density (e.g. $H_2PO_4^-$, HSO_4^-, HCO_3^-, I^-, Cl^-, NO_3^-, NH_4^+, Cs^+, K^+ and tetramethylammonium ions; exhibiting weaker interactions with water than water with itself), are chaotropes whereas small or multiply-charged ions, with high charge density, are kosmotropes (e.g. SO_4^{2-}, HPO_4^{2-}, Mg^{2+}, Ca^{2+}, Li^+, Na^+, H^+, OH^- and HPO_4^{2-}, exhibiting stronger interactions with water molecules than water with itself).

From the above it clearly can be seen that sodium chloride cannot be classified as the "classic" chaotrope substance - on the contrary sodium chloride seems more to be qualified as a kosmotrope in the sense of the Hofmeister series.

In particular regard to this situation, appellant takes liberty to refer to the fact that the examiner takes in the Office communication dated October 26, 2001 the point of view, that "sodium chloride clearly should be a chaotrope" [page 10, lines 19 to 25]:

> The Henco reference does not make specific reference to a chaotropic agent. In addition, the elution buffers used in Henco contain various proportions of NaCl, a compound notorious well-known in the art to alter the structure of water, and therefore NaCl must be also a chaotropic agent.

This is a fatal misinterpretation in the light of the Hofmeister series.

However, the question raised by the Examiner in the Office communication (dated September 17, 2002) focuses on the use of urea as a chaotropic compound. The Examiner relies on two

additional references. From appellants' point of view it cannot be seen that these two references would be of any help with regard to the proceedings or how these references support the Examiner's position.

Henco is concerned with a method for the separation of long-chain nucleic acids from other substances in solutions containing nucleic acids and other materials, comprising fixing long-chain nucleic acids in a nucleic acid-containing solution in a porous matrix, washing the porous matrix to separate the other substances from the long-chain nucleic acids, and removing the fixed long-chain nucleic acids from the porous matrix.

The object of Henco [column 4, lines 38 to 61] (*emphasis added*) is to provide

a process for removing long-chain nucleic acids from tissues and body liquids which:

a) in a similar manner allows the nucleic acids to be extracted and concentrated from various starting materials, such as tissue, blood, sputum, cell cultures, bacteria, fungi, renal and fecal excrements, as well as from vegetable tissue from callus cultures, roots, etc.;
b) requires no long-time centrifugation steps, and more specifically no ultracentrifugation;
c) can be carried out without expensive equipment, and more specifically without refrigerated centrifuges and ultracentrifuges, and without using valuable material, such as caesium chloride for density gradients or rotor insertions for one-time use;
d) ensures high purity of the nucleic acid to be attained.
e) works without a phenolic extraction step; and
f) is suitable for being automated;

and by means of extraction of the long-chain nucleic acid, separates mixtures of long-chain nucleic acids and other materials, such as those obtained when products are biotechnologically produced.

Clearly, Henco refers to the use of urea at different places of the disclosure, mostly in cases where the biological samples for the long chain nucleic acid isolation are bacteria or viruses. For example:

Column 8, lines 59 to 63:

> The method according to the invention utilizes the described porous matrix by lysing the CMV viruses in situ by addition of urea, detergent and buffer, whereupon the DNA (130 to 150 × 10^6 Dalton) is released.

Column 12, lines 33 to 38 [Example 2]:

> Upon simultaneous addition of 4 M of urea, the DNA of the phages is released and, by means of another filtration through the cartridge, specifically adsorbed on the anion exchanger. Then the cartridge is washed with 0.8 M NaCl, 50 mM Tris-HCl buffer, pH 7.5, 1mM EDTA, and the DNA is eluted with about 1 ml of 1.2 M NaCl, 50 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA.

Column 12, lines 63 to 69 [Example 3]:

> *"...and the phage pellet is dissolved in 20 µl of 10 mM Tris, 1 mM EDTA, pH 7.5. Another part by volume of extraction buffer (2% Triton X-100®, 7 M urea, 100 mM EDTA, pH 7.5) is added, and the mixture is heated at 50 °C for 15 minutes to release the single-stranded DNA."*

Column 13, lines 14 to 24 [Example 4]:

> *The isolation of cellular DNA from sperm is carried out as follows:*
>
> *One hundred µl of sperm are suspended in 1 ml of 500 mM NaCl, 10 mM EDTA 40 mM DTE, 10 mM Tris-HCl buffer, pH 7.5, 1% Triton, 4 M urea and 20 µg/ml of proteinase K and incubated at 37 °C for 2 hours. After centrifugation at about 5000 g for 5 minutes, the supernatant is passed through the separating gel in a cartridge. The flow velocity of the supernatant through the cartridge is about 1 ml/min.*

Column 13, lines 64 to 68 [Example 7]:

The preparation of CMV (cytomegalovirus) DNA from urine is carried out as follows:

CMV viruses are lysed in situ upon addition of 4 M urea, 1% Triton, 500 mM NaCl, 50 mM Tris-HCl buffer, pH 7.5

It must be stated that all these citations clearly show that Henco uses urea only for the purpose of lysis (as was already pointed out in our facsimile letter dated October 8, 1999). There is no hint to use urea as a chaotropic substance in an elution buffer or for the purpose to wash nucleic acid adsorbed on a matrix. On the contrary, Henco discloses that the addition of urea to the loading buffer (i.e., before the adsorption/binding of the nucleic acid) has no effect the binding behavior of the long chain DNA.

This can clearly be seen from the description column 7, lines 8 to 11:

The addition of urea to the loading buffer does not affect the binding behavior of the long-chain DNA, while it optimizes the separation efficiency with respect to proteins.

As can be seen, the only advantageous effect of the addition of urea results in a better separation efficiency with regard to the proteins. However, this is of no help at all with regard to the solution of the problem underlying the presently claimed invention. Even if one would like to argue that the addition of urea to the loading buffer has positive effects with regard to the separation of nucleic acid from proteins during the following elution from the porous matrix and if - assuming arguendo - one would like to follow the line of argumentation of the Examiner that urea implicitly mans the use of any substance which destroys the order of water in which it is dissolved (i.e., a chaotrope) the advantageous effect of protein separation will only show relevant for unpurified lysates.

It must be noted that according to the presently claimed invention the mixture resulting from step b) and being the starting material of step c) is already purified and, thus, is free from proteins. Accordingly it cannot be seen that the teaching of Henco is of any help for the solution of the problem of the presently claimed invention because the use of urea or a chaotrope at such a late step is simply superfluous in the light of the disclosure of Little.

However, it cannot be seen that the use of urea as a lysis agent leads those skilled in the art to use chaotropic substances in a washing buffer, to wash nucleic acids which are bound on a matrix.

It should be born in mind that a skilled reader will study a document in a practical manner and in an afford to make sense of it. Thus, the skilled reader will discard any possible interpretations of the document which are illogical, impractical or fanciful. If the Henco citations is read in this manner, it will immediately be evident that Henco is no good point to start from if a skilled man is going to solve the problem underlying the presently claimed invention.

Little is concerned with a process for the purification of plasmid and other DNA, both single-stranded and double-stranded, by immobilizing the DNA onto diatomaceous earth in the presence of a chaotropic agent and eluting the DNA with water or low salt buffer and a process for the immobilization of DNA onto diatomaceous earth in the presence of a chaotropic agent.

The invention is based on the finding that diatomaceous earth is useful for the purification of plasmid and other DNA by immobilizing the DNA onto the diatomaceous earth particles in the presence of a chaotropic agent, following by elution of the DNA with water or low salt buffer.

More particularly, the Little citation discloses a process for the purification of plasmid DNA comprising the following steps:

a) immobilizing the DNA onto diatomaceous earth in the presence of a chaotropic agent;

b) washing the resulting diatomaceous earth-bound DNA with an alcohol-containing buffer;

c) removing the alcohol-containing buffer; and

d) eluting the DNA in a low salt buffer or in water.

As already mentioned above, the invention is also directed generally to the immobilization of DNA onto diatomaceous earth which comprises contacting the DNA with the diatomaceous earth in the presence of a chaotropic agent.

According to Little (column 3, lines 36 to 43) a chaotropic substance is a

> substance that enhances the partitioning of nonpolar molecules from a nonaqueous to an aqueous phase as a result of the disruptive effect that the substance has on the structure of water. Examples of chaotropic agents include sodium iodide, sodium perchlorate and sodium trichloroacetate.

First, it should be noted that Little does not identify sodium chloride among the exemplary "chaotropic agents." Secondly, while Little does use a chaotropic substance to bind nucleic acids to a diatomaceous earth matrix, the reference (Little column 4, lines 21 to 35) also uses a chaotropic binding buffer, i.e.:

> An example of an alcohol-containing wash buffer comprises: 20.0 mM Tris-Cl pH 7.5, 20 mM EDTA, 0.4 M NaCl, and 50% v/v ethanol. This buffer will be abbreviated herein as "50% ethanol buffer" or "50% washing buffer.
>
> In order to lower the RNA and protein concentration in plasmid lysates, it is necessary to perform a sufficient number of washes using the chaotrope binding buffer and the 50% washing buffer. The amount of RNA and protein remaining is indirectly proportional to the number of volume washes performed on the diatomaceous earth pellet, membrane or column. Generally, about three washes of

> each buffer is sufficient to lower the RNA and protein concentrations to acceptable levels.

Accordingly, the "washes"—using the chaotropic and washing buffers—are necessary to lower the RNA and protein concentrations in plasmic lysates. However, similar washes are, also, necessary when using complex, non-purified lysates as starting materials.

On the other hand it must, again, be noted that according to the presently claimed invention the mixture resulting from step b) and being the starting material for step c) is purified and, thus, is free from RNA and proteins.

Moreover, it should be noted that Little's intention with regard to the elution of the DNA was to optimize the yield of DNA to be recovered [column 4, lines 36 to 46]:

> The efficiency of release of immobilized DNA from the diatomaceous earth pellet, membrane or column will be proportional to the ratio of the volume of low-salt buffer or water added to the volume of the pellet, membrane or column. Thus, with a 5 μL diatomaceous earth pellet, for example, 5 μL of buffer or water (1 volume) will extract about 50% of the DNA. Likewise, 10 volumes of buffer or water added per volume of pellet will permit the recovery of >90% of the DNA. However, it should be kept in mind that the more buffer or water added, the more dilute the eluted DNA.

Accordingly, Little does not face at all the problem to isolate the DNA in a solution having a low salt concentrate.

Thus, appellants submit that the skilled artisan would not find Little a good place to start from when looking for teaching to solve the problem of the presently claimed invention. Furthermore, there was absolutely no motivation for a man skilled in the art to combine the teaching

of both references - which both are concerned with DNA isolation from crude starting material - to reach the two step process of the presently claimed invention.

Even if - what is denied - one skilled in the art would have taken into account such a combination, it is clear - as discussed above - that he has to be inventive to find all the new parameters for such a two step isolation. The basic knowledge that urea does belong to the group of chaotropic substances would be of no or no essential help to arrive at a solution of the problem underlying the presently claimed invention.

The Examiner argues (on page 13, lines 14 to 22, of the final action):

> Therefore, Henco '426 use a combination of "urea, detergent and buffer" to effect lysis of CMV viruses (column 8, lines 60-62) is plainly an example wher etow of the three components of the lysis buffer are chaotripic (urea and detergent), a reality which hte lsysi buffer relies on the effect on cell wall lysis. Examiner concludes that applicant's argument is not convincing because there is no requirement that the prior art use any particular term to specify in the absence of the presence of specific term.

Appellant submits that this statement is incorrect. As was shown above, urea, when it was used in Henco, it was used in all cases in the lysis step. According to this disclosure it is no doubt true that the teaching of Henco clearly is, to use urea for the lysis (and not for the elution). The side effect of the better separation of the proteins from the nucleic acid is - as was shown of no relevance for the use of some special chaotropic substances (as listed in claim 116) according to the presently claimed invention.

What the Examiner tries is to take the word "urea" completely out of context of the teaching of Henco, to search for a specific inherent feature (which is of no meaning for the use disclosed by

Henco) and in the last step – after generalization (chaotropic substances) to find – according to his opinion – an appropriate other feature and use of such class of compounds (as washing buffers) and to build up a very artificial and highly impermissible hindsight reconstruction only based on this new feature.

(B) Claims 122-126 and 129-136

As indicated above, each of claims 122-126 and 129-137 is independently patentable. No prior art disclosure is relied on to meet the additional claim limitations recited in each of these claims. Instead, according to the Examiner (final Office Action, page 4):

> The specific details of washing steps, the timing of steps, the specific selection of wash solution contents, and the physical characteristics of the anion exchange resin and mineral adsorbent (e.g., particle diameter, pore size, etc.) are deemed to be variables clearly within the purview of ordinary practitioner seeking to optimize the Henco and Little process steps for a specific situation. Therefore, the details of adsorbent choice or other standard performance parameters (e.g., the frequency of washes, the variation of ionic strength in wash solutions, etc.) are deemed to be the kind of variables properly within the realm of routine experimentation by an ordinary practitioner in the course of optimizing the process steps disclosed in the prior art of record. For these reasons, the instant claims, insofar as they are directed to routine changes in experimental details of the kind noted above, are deemed to lack an adequate basis for a finding of patentable distinction for any variation of the instant claimed process, as such variations are deemed to have been properly included within the scope of the noted prior art.

"Reliance on *per se* rules of obviousness is legally incorrect and must cease." *In re Ochiai*, 37 USPQ2d 1127, 1129 (Fed. Cir. 1995). If the prior art fails to disclose a rationale for varying

parameters to be result effective, it can not have been obvious to choose the claimed parameter. *In re Antonie*, 195 USPQ 6 (CCPA 1977). Obviousness cannot be based on speculation.

> The examiner should be aware that "deeming" does not discharge him from the burden of providing the requisite factual basis and establishing the requisite motivation to support the conclusion of obviousness. . . . The examiner's reference to unidentified phantom prior art techniques . . . falls short of the mark.

Ex parte Stern, 13 USPQ2d 1379, 1382 (BPA&I 1989).

> Whether the changes form the prior art are "minor", as . . . [patent challenger] argues, the changes must be evaluated in terms of the whole invention, including whether the prior art provides any teaching or suggestion to one of ordinary skill in the art to make the changes that would produce the . . . [claimed] method and device.

Northern Telecom, Inc. v. Datapoint Corporation, 15 USPQ2d 1321, 1324 (Fed. Cir. 1990). Differences between the claims and the prior art do not amount to "an obvious design choice," when the differences "achieve different purposes." *In re Gal*, 25 USPQ2d 1076, 1078 (Fed. Cir. 1992). Where the *optimization* of a claim variable was not recognized in the art as effecting the claimed result, the result is unobvious. *Antonie*, 195 USPQ at 8. That a difference with the prior art amounts to an alleged "optimal condition . . . is not a substitute for some teaching or suggestion supporting an obviousness rejection." *Rijckaert*, 28 USPQ2d at 1957. When obviousness of a claim limitation is grounded on its allegedly being "old and well known in the art . . . as a means of optimization which is highly desirable," the "ground of rejection is simply inadequate on its face . . . because the cited references do not support each limitation of [the] claim." *In re Thrift*, 63 USPQ2d 2002, 2008 (Fed. Cir. 2002).

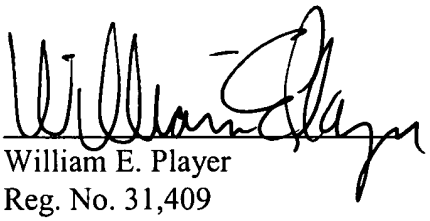
* * *

CONCLUSION

For the foregoing reasons, reversal of all appealed rejections of record is requested.

Respectfully submitted,

JACOBSON HOLMAN PLLC

By: 
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(8) Claims Appendix

Claims 1-119 (cancelled)

120. A process for the isolation and purification of nucleic acids from cells comprising, in two separation/purification stages, the steps of:

i) in a first separation/purification stage,

a) digesting the cells containing nucleic acids, removing cell debris and thereafter subjecting the nucleic acids to anion exchange against an anion exchanger in a first buffer solution, which has a low ionic strength,

b) desorbing the nucleic acids from the anion exchanger by applying a second buffer solution, which has a higher ionic strength than the first buffer solution, effecting purified nucleic acids in the second buffer solution; and

ii) in a second separation/purification stage,

c) adsorbing the separation/purified nucleic acids in the second buffer solution onto the surface of a mineral support material, optionally in the presence of lower alcohols, poly(ethylene glycol), or a mixture thereof, and

d) desorbing the nucleic acids from the mineral support material by applying an eluant, wherein the eluant is water or a third buffer solution, which has an ionic strength lower than the second buffer solution, effecting twice-purified nucleic acids.

121. The process according to claim 101, wherein the stages i) and ii) are carried out in immediate succession.

122. The process according to claim 120, further comprising the step of, prior to the digesting step, subjecting the cells to centrifugation or filtration in order to remove undissolved components.

123. The process according to claim 120 further comprising, between the steps a) and b), one or more washing steps by applying a fourth buffer solution, which has a low ionic strength, optionally increasing ionic strength per washing step.

124. The process according to claim 120 further comprising, between the steps c) and d), one or more washing steps by applying a fifth buffer solution, which has an ionic strength higher than the first buffer solution.

125. The process according to claim 120 further comprising, between the steps c) and d), at least one washing step by applying an aqueous alcoholic solution.

126. The process according to claim 120 further comprising, between the steps c) and d), a washing step by applying a solution having an ionic strength corresponding to a 1.5 molar sodium perchlorate solution and a pH of 5.

127. The process according to claim 120, wherein the isolated and purified nucleic acid has from 10 nucleotides to 200,000 nucleotides.

128. The process according to claim 120, wherein the mineral support material is silica gel, glass, zeolite, aluminum oxide, titanium dioxide, zirconium dioxide, kaolin, or diatomacae.

129. The process according to claim 120, wherein the anion exchanger has a porous or non-porous matrix having a particle size of from 1 to 250 μm.

130 The process according to claim 120, wherein the anion exchanger has a porous or non-porous matrix having a particle size of from 10 to 30 μm.

131. The process according to claim 120, wherein the mineral support is silica gel, in suspension, having a particle size of from 1 to 250 μm.

132. The process according to claim 120, wherein the mineral support is silica gel, in suspension, having a particle size of from 1 to 5 μm.

133. The process according to claim 120, wherein the anion exchanger has a particle size of from 1 to 250 μm and a pore diameter of from 1 to 2,500 nm.

134. The process according to claim 120, wherein the anion exchanger has a particle size of from 10 to 100 μm and a pore diameter of from 1 to 2,500 nm.

135. The process according to claim 120, wherein the anion exchanger has a particle size of from 1 to 250 μm and a pore diameter of from 100 to 400 nm.

136. The process of claim 125, wherein the aqueous alcoholic solution includes from 1 to 7 M sodium perchlorate, from 1 to 7 M guanidine-HCl, from 1 to 5 M sodium chloride, from 1 to 6 M sodium iodide, or 1 M sodium chloride in a 20% alcoholic solution wherein the alcoholic portion of the alcoholic solution is selected from the group consisting of ethanol, propanol, isopropanol, butanol, poly(ethylene glycol), and mixtures thereof.

137. The process of claim 120, wherein the eluant is a buffer solution that comprises water and Tris at a pH value of from 5 to 9.

138. The process of claim 120, whereby the nucleic acids are plasmid or genomic DNA.

(9) Evidence Appendix.

1. References relied on by the examiner to support the final rejection under 35 USC.

   - US 5,057,426 (Henco).
   - US 5,075,430 (Little).
   - *International Dictionary of Medicine and Biology, 1*, 1986, page 522 (*International Dictionary*).
   - *Nucleic Acid Hybridisation - A Practical Approach*, 1985, pages 64, 65, and 235 (Hames).

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Chantemesse [André *Chantemesse*, French bacteriologist, 1851–1919] See under VACCINE.

Chaoborus A genus of nonbloodsucking midges or gnats which are important as predators of mosquitoes. The Clear Lake gnat of California, *C. asticopus* (or *C. lacustris*) can be a nuisance when it swarms around lights at night. Also called *Corethra*.

Chaos chaos *PELOMYXA CAROLINENSIS.*

chaotropic [Gk *chao(s)* unformed mass + -TROPIC¹] Denoting a substance that destroys the order of water when dissolved in it and thereby raises the solubility of hydrophobic substances in the solution. Chaotropic agents are often large anions, such as thiocyanate and perchlorate. A chaotropic series is a listing of substances in the order of their chaotropic power.

Chaoul [Henri *Chaoul*, German radiologist, 1888–1964] See under THERAPY, TUBE.

chap [Middle English *chappen*] To develop cracks or fissures in the skin as a result of dehydration. This tends to occur particularly in cold and windy conditions.

chaparro amargoso A plant of the species *Castela nicholsoni*, in the Simaroubaceae family, which has been used in Mexico in the treatment of protozoan infections.

Chapman [John *Chapman*, English physician, 1822–1894] See under BAG.

chappa [Sudanese-Guinean] A local manifestation of yaws in southern Nigeria, including Lagos, in which severe muscular and articular pains give way to development of multiple nodules, over which ulcers, without abscess formation, develop. Bones are ultimately involved.

Chaput [Henri *Chaput*, French surgeon, 1857–1919] See under METHOD.

character [L (from Gk *charaktēr*, impress or stamp on a person or thing, likeness), a mark set upon any thing, a style of writing or speaking] 1 A distinctive feature, trait, or property; attribute, as *secondary sex characters*. Also called *characteristic*. 2 The distinctive attributes of a person, including innate endowment and constitutional factors as well as the habitual attitudes and traits that have developed as a result of experience or training, the totality of a person's relationship to his environment and his ego-syntonic style of relating to others, and the constellation of defense mechanisms that a person automatically and customarily employs to maintain psychosocial stability. Also called *personality, personality type*. • Historically, *character* implied an emphasis on the nature of the subject, while *personality* emphasized experiential, interpersonal factors.

acquired character A phenotype produced by environmental influences.

anal character COMPULSIVE PERSONALITY.

authoritarian character A character marked by belief in authority as a source of truth, the requiring of unquestioning subservience to and identification with authority, and such personality traits as rigidity, conventionality, detestation of weakness, and intolerance of ambiguity.

canalized character A phenotypic trait little affected by environmental or genetic perturbations acting on the organism during development.

compound character A phenotype produced by two or more genes.

dominant character DOMINANT TRAIT.

epileptic character A group of behavioral and personality characteristics once believed to be identifiable in epileptic subjects. Patients with psychosis, and with affective and personality disorders, may suffer from epilepsy, which can also be a manifestation of organic brain disease which may in turn cause behavioral changes. Patients with severe and frequent attacks of epilepsy may also suffer brain damage as a direct consequence of the attacks. Also called *epileptic constitution* (obsolete), *epileptic temperament, glischroidia* (obsolete), *epileptoid personality, explosive personality*. • Even though most authorities agree that no specifically epileptic character can be identified, the term continues to be used and implies one or more of the following features: rigidity, egocentricity, religiosity, and explosive outbursts of emotion.

exploitative character A personality type characterized by the attempt to manipulate situations perceived as dangerous through cajolery, flattery, aggressive counterthreats, or intimidation. • The term was used by Erich Fromm.

genital character A character in which the oral, anal, and phallic phases of libido development dovetail into the concomitantly developing object relationships to achieve the adult capacity for impersonal, non-narcissistic, and unambivalent love of the object and unhampered orgastic sexuality.

mendelian character A phenotype that is determined solely or predominantly by a gene or genes at a specific locus on the chromosome. In humans, such characters may be autosomal dominant, autosomal recessive, or X-linked. Also called *unit character*.

monogenic character Any phenotypic feature determined by a single gene locus, the inheritance of such characters being in accord with mendelian laws. Also called *single gene trait*.

oral character A character with a predominance of traits originating in the vicissitudes of libido development at the oral phase. Traits stemming from the earlier oral receptive (preambivalent) phase include optimism, generosity, and an expectation that others will take care of one. The later oral aggressive (oral incorporative) phase leads to aggressiveness, envy, ambition, vindictiveness, and a tendency to exploit or manipulate others. Also called *oral personality*.

oral aggressive character A personality type characterized by aggressiveness, competitiveness, envy, and an exploitative character. In psychoanalytic theory, the oral aggressive character results from inadequate satisfaction of the child during the oral stage of psychosexual development.

paranoid character A character marked by mistrust of others, suspiciousness, hypersensitivity to criticism, readiness to take offense, and a tendency to use projection mechanisms in dealing with reality. Also called *paranoid personality*.

polygenic character Any phenotypic feature determined by many genes. Such characters are often quantitatively variable and their inheritance does not follow mendelian laws.

primary sex characters The distinctive gonadal or genital characters of each sex, as the testis in the male and the ovary in the female. They are the gametogenic and hormone-secreting organs of the male or female sex which are responsible for the development of secondary sex characters.

psychotic character A character which typifies an individual with residual or latent symptoms of schizophrenia but with no gross break with reality. An imprecise usage.

quantitative character A polygenic character whose variability can be specified metrically. It is inherited in a multifactorial mode, with the genetic component determined by many genes.

receptive character A character distinguishing an individual who is characterized by optimism, generosity, and a feeling that others will take care of that person with reactive pessimism and self-doubt when those wants are not fulfilled. In some typologies, the receptive character is interpreted as a sublimation of the preambivalent phase in libidinal development.

recessive character RECESSIVE TRAIT.

secondary sex characters The extragonadal characters specific for maleness or femaleness, such as phallic growth and breast development; the features other than those involving gametogenesis and secretion of the sex hormones.

Nucleic acid hybridisation

a practical approach

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Cover illustration. On the left is an autoradiograph of λgt11 recombinants containing *Dictyostelium discoideum* genomic DNA inserts, screened on nitrocellulose filters with a nick-translated 4.1 kb repetitive genomic fragment by the method of Benton and Davis (see Chapter 5). On the right is an autoradiograph of part of *Drosophila melanogaster* polytene chromosome 2R (stained with Giemsa) showing multiple sites of *in situ* hybridisation by the mobile element pDmI 137 [Dawid *et al.* (1981) Cell **25**, 399]; magnification 1300 x. The photographs were kindly supplied by Ms. P. Jagger and Mr. D.P. Ramji (Department of Biochemistry, University of Leeds, UK) and Dr. M.L. Pardue (M.I.T., Cambridge, USA), respectively.

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son (1) who showed that the rate was strongly dependent on sodium ion concentration at least up to 3.2 M. At concentrations up to 0.2 M the rate has been shown to be proportional to the cube of the ionic strength (23). Comparisons of reaction rates at different salt concentrations can be greatly simplified by use of relative reaction rates which Britten *et al.* (17) computed from an empirical formula (see *Table 3*). It should be noted that the original data on which this table was based were obtained at 25°C below the melting temperature of DNA at each salt concentration. Hence the relative rates of reaction in this table represent optimal rates and may not apply directly to reactions which are carried out at sub-optimal temperatures. Furthermore the formula applies to measurements of DNA reannealing using hydroxyapatite for analysis. However, the table will still apply to measurements of relative rates.

The effect of ionic strength on rate of RNA-DNA hybridisation has not been studied in detail. However, it does appear that RNA-DNA hybridisation is also strongly dependent on ionic strength. For example, Nygaard and Hall (24) demonstrated a 5- to 6-fold increase in the rate of hybridisation of phage T2 RNA to T2 DNA by increasing the ionic strength from 0.2 M to 1.5 M NaCl.

6.4 Viscosity

In considering the effect of viscosity on the rate of reaction it is important to distinguish between microscopic and macroscopic viscosity. Microscopic viscosity refers to the micro-environment around the DNA bases and is commonly altered by the addition of sucrose, glycerol or sodium perchlorate. The macroscopic viscosity is dependent on the presence of polymers (including DNA) which will have no effect on the micro-environment. Thus a measurement of viscosity with a viscometer will include both micro- and macroscopic viscosity if there are polymers present.

Thrower and Peacocke (25) and Subirana and Doty (26) observed that increasing the microscopic viscosity with sucrose decreased DNA renaturation rates. A more detailed analysis (1) showed that in sucrose, glycol, ethylene glycol and sodium perchlorate the optimal rate of DNA renaturation is inversely proportional to the microscopic viscosity. This observation of the effect of microscopic viscosity has been confirmed by Chang *et al.* (27) who extended their studies to include the effect of macroscopic viscosity on DNA renaturation. It was shown that in a 5.7% Ficoll solution (a neutral polymer) the rate of phage T4 DNA renaturation was increased by 50%. Similarly in a 2% solution of the anionic polymer, sodium dextran sulphate, the rate of phage T2 DNA renaturation was increased by 4-fold, despite a 6-fold increase in the macroscopic viscosity. This increase in reaction rate was attributed to the exclusion of DNA from a substantial volume of the solution by the added polymers, resulting in an increase in the effective DNA concentration. Dextran sulphate is now widely used to accelerate reactions to filter-bound DNA (see Chapter 4).

6.5 Denaturing Agents

The optimal temperatures for nucleic acid reassociation in aqueous salt solutions lie in the range 60 – 75°C. However, the extended incubation at such temperatures which is required for reassociation of complex eukaryotic nucleic acid can lead to a considerable amount of thermal strand scission. Hence it is desirable to reduce the temperature whilst

maintaining the stringency of the nucleic acid interaction. This can be achieved by introducing a reagent, such as formamide, which destabilises double-stranded nucleic acid. Thus, a 1% increase in formamide concentration lowers the T_m of native duplex DNA by 0.72°C. In contrast, for RNA-DNA hybrids the relationship between formamide concentration and the depression of T_m is not linear (see Chapter 4, section 4). However, altered conditions generally result in a reduction in reaction rate and therefore the experimenter must decide whether the benefit of a reduced rate of degradation outweighs the disadvantage of a reduced reaction rate. Although formamide is the most popular and well characterised reagent used to reduce reaction temperatures (28,29), a variety of other denaturing solvents have been tried including ethylene glycol (1), sodium perchlorate (27), tetramethylammonium chloride and tetraethylammonium chloride (27) and urea (17,30).

The most systematic study of the effect of formamide on the reassociation rate of DNA was carried out by Hutton (30). It was shown that the characteristic bell-shaped dependence of renaturation rate on temperature is maintained in solutions containing formamide. Furthermore, increasing the formamide concentration decreases the optimal renaturation rate by 1.1% per 1% of formamide. It was also demonstrated that the reduction in rate can be accounted for by the increased microviscosity of the solution. A comparison of the rate of degradation of DNA in an aqueous high temperature system with a formamide low temperature system showed that the reduced reaction rate was more than compensated for by a lower rate of degradation.

Schmeckpeper and Smith (29) found that the presence of 50% formamide reduces the rate of RNA-DNA hybridisation by a factor of 0.25 compared with the reaction rate in an aqueous solution at a similar stringency. The stability of RNA-DNA hybrids is greater in 50% formamide than that of DNA duplexes of similar base composition (22). This has been exploited for the purpose of electron microscopy to allow RNA-DNA hybrids to form whilst preventing DNA duplex formation. Using solution reactions, Vogelstein and Gillespie (31) and Casey and Davidson (32) showed that 70% formamide does not permit DNA reannealing at temperatures between 41 and 50°C but does allow almost complete RNA-DNA hybridisation.

6.6 Mismatching

It is well established that the presence of mismatched base pairs reduces the thermal stability of DNA duplex molecules. It is therefore to be expected that the imperfect sequence complementarity will also have an effect on the rate of reassociation. This is particularly important where reassociation is used to study the divergence of the DNAs of related species.

The extent of mismatching in a DNA duplex is experimentally determined by measuring the reduction in T_m of the mismatched DNA compared with a modified control DNA. Hence the effect of mismatching on the rate of DNA reannealing is usually quoted in terms of effective reduction in T_m. From several studies (20,27,33,34) it is clear that mismatching of DNA which results in a decrease in the T_m of about 15°C will reduce the reannealing rate by a factor of 2.

6.7 Temperature

The dependence of DNA reassociation rate on incubation temperature was first studied

INDEX

(10) Related Proceedings Appendix.

None.

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